

[illegible]

Attorney
Docket: 01/22289

In the latest Official action, the Examiner has rejected claims 1-7, 12-18, 22-28, 32-38, 42-47 and 84-91 under 35 U.S.C. § 112, first paragraph as containing subject matter which was not described in the specification in such a way as to enable one of ordinary skill in the art to make and/or use the invention. The

Examiner has stated that while the specification clearly discloses the successful isolation of a novel flavanone-7-*O*-glucoside-2''-*O*-rhamnosyl-transferase gene from Pummelo using degenerate PCR primers designed from fragments of the digested enzyme, the instant specification does not describe any other sequences that would encode a polypeptide having flavanone-7-*O*-glucoside-2''-*O*-rhamnosyl-transferase activity, or which sequences of the peptide are conserved or known motifs and would allow for isolation of functional equivalents. Thus, the Examiner has asserted that the instant specification does not describe the genus comprising functional embodiments that fall within the range of the invention as claimed.

I would like to reemphasize that the present invention is of a novel, unique 1-2-rhamnosyl-transferase gene that was isolated and cloned from Pummelo young leaf mRNA by RT-PCR using unique gene specific PCR, and the recombinant protein product thereof (SEQ ID NO: 21). The specific flavanone-7-*O*-glucoside-2''-*O*-rhamnosyl-transferase (α -1-2 rhamnosyl transferase) activity of the polypeptide encoded by the polynucleotides of the present invention had not been demonstrated for any other known sequences at the time the application was filed. Thus, the coding sequence of the present invention is unique to this specific enzyme, and it was expected that homologues having a high degree of nucleic acid or amino acid sequence homology to SEQ ID NOs: 20 and 21 would constitute functional equivalents of the Pummelo flavanone-7-*O*-glucoside-2''-*O*-rhamnosyl-transferase gene and polypeptide, respectively.

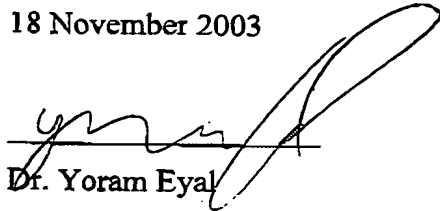
In that context, I would like to report the recent discovery of a previously unknown sequence having a high degree of homology to the Pummelo flavanone-7-*O*-glucoside-2''-*O*-rhamnosyl-transferase gene coding sequence. (SEQ ID NO:20). I performed a homology search according to the EMBL BLAST2.0 search protocol available at www.ch.embnet.org using the coding sequence of Pummelo flavanone-7-*O*-glucoside-2''-*O*-rhamnosyl-transferase gene as described in the present invention (SEQ ID NO:20), and an EST database (dbEST) and identified a homologous sequence of an EST from a *Poncirus trifoliata* (Japanese- or bitter orange) cDNA library (CF 419914, submitted September 3, 2003), having a 96% sequence identity with a large portion (nucleotides 685-1208) of the Pummelo flavanone-7-*O*-glucoside-2''-*O*-rhamnosyl-transferase gene coding sequence (SEQ

ID NO:20) (see Appendix I and II, enclosed herewith). When compared using the EMBL align amino acid alignment program, it was revealed that the translated amino acid sequence of the *P. trifoliata* EST also displays a high (95%) identity to the amino acid sequence of the Pummelo flavanone-7-O-glucoside-2''-O-rhamnosyl-transferase polypeptide (SEQ ID NO:21) (see amino acid alignment, Appendix II, enclosed herein). Searching for protein domain homology using the NCBI Conserved Domain Search program available at www.ncbi.nlm.nih.gov, it was revealed that the translation product of the *P. trifoliata* EST (CF 419914) exhibits only very low homology (15-36%) to any sequences other than SEQ ID NO:21. More significantly, however, it was revealed that the translation product of the *P. trifoliata* EST shows some limited homology to polypeptide domains common to glycosyl-transferases, such as UDP glucuronosyl and glucosyl transferases, and UDP-N-acetylglucosamine:LPS-N-acetylglucosamine transferase (see Appendix IV), indicating a functional homology as well as structural homology to Pummelo flavanone-7-O-glucoside-2''-O-rhamnosyl-transferase enzyme.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

18 November 2003



Dr. Yoram Eyal

Enc.:

Curriculum Vitae of Dr. Yoram Eyal
Appendices I, II, III and IV

Yoram Eval – BRIEF CURRICULUM VITAE

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EDUCATION

1982-1985 Hebrew University - B. Sc. in Life Sciences
1985-1987 Weizmann Institute of Science - M. Sc. in Plant molecular genetics & physiology.
1987-1992 Weizmann Institute of Science - Ph. D. in Plant molecular genetics & physiology.

EMPLOYMENT AND STUDY LEAVES

1992 - 1995 Postdoctoral researcher at the PGEC, University of California, Berkeley CA.
1996 - Researcher at the Institute of Horticulture, The Volcani Center, ARO, Israel

AWARDS AND SELECTED RESEARCH GRANTS

1985 - 1987 MSc scholarship, Weizmann Institute of Science
1987 - 1992 PhD scholarship, Weizmann Institute of Science
1992 - 1994 Postdoctoral fellowship, BARD
1996 - 1999 BARD: Pollination compatibility
1998 - 2002 BARD: Metabolic engineering of flavonoid composition in grapefruit
1999 - 2002 Ministry of Science: Biotechnology of terpenoid natural compounds
2001 - 2004 Ministry of Agriculture: The role of Chlorophyllase in chlorophyll catabolism

DEGREES SUPERVISED

5 M.Sc. and 2 Ph.D students

PUBLICATIONS

18 peer-reviewed journal articles and invited reviews; 4 patents

AREAS OF EXPERTISE

Plant physiology, biochemistry and molecular biology

Specifically:

Biosynthesis of flavonoid and terpenoid flavor and aroma compounds in citrus
Metabolic engineering of flavonoid and terpenoid biosynthesis pathways
Chlorophyll catabolism regulation

Appendix I

Alignments

☐ >gi|34432615|gb|CF419914.1|CF419914 USDA-FP_117000-855 Immature Ovaries
from field collected Flying
Dragon Trifoliate Orange Poncirus trifoliata cDNA clone
TFF-18_H04 5'.
Length = 525

Score = 910 bits (459), Expect = 0.0
Identities = 507/524 (96%)
Strand = Plus / Plus

```
Query: 685 gaaataattccagtagggcctctaataccaagaacctaccttcaaggtagatgatacaaag 744
          |||
Sbjct: 2 gaaataattccagtagggcctctaataccaagaacctactttcaaggaagatgatacaaag 61

Query: 745 atcatggactggctgagccaaaaggagcctcgttcagtcgtgtatgcatcctttggcagt 804
          |||
Sbjct: 62 atcatggactggctgagccaaaaggagcctcgttcagtcgtgtatgcatcctttggcagt 121

Query: 805 gagtactttccttccacggatgaaatacatgacatagctattgggttattgctcaccgag 864
          |||
Sbjct: 122 gagtactttccttccaaggatgaaatacatgagatagctagtggattattgctcagcgag 181

Query: 865 gttaattttatatgggctttcagattacatcctgatgagaaaatgacgatagaggaagca 924
          |||
Sbjct: 182 gttaattttatatgggctttcagattacatcctgatgaaaaatgactatcgaggaggca 241

Query: 925 ctgcctcagggctttgctgaggagattgaaaggaataataagggaatgatagtacaaggt 984
          |||
Sbjct: 242 ctgcctcagggctttgctgaggagattgaaaggaataataagggaatgatagtacaaggt 301

Query: 985 tgggttccgcaggctaaaattttaaggcatggaagcatcgcggtttttgagtcattgt 1044
          |||
Sbjct: 302 tgggttccgcaggctaaaattttaaggcatggaagcatcgcggtttttgagtcattgt 361

Query: 1045 gggttggggctcggtgggttgaggggatggttttcgggggtaccaatcataggtgtgccaatg 1104
          |||
Sbjct: 362 gggttggggctcggttagttgaggggatggttttcgggggtaccaatcataggtgtgccaatg 421

Query: 1105 gcatatgagcagccaagcaatgccaagggtggtggttgacaatggtatgggcatggtcggt 1164
          |||
Sbjct: 422 gcatatgagcagccaagcaatgccaaggnggtggttgacaatggtatgggcatggtcggt 481

Query: 1165 ccaagagataagatcaatcaaagacttgaggagaggaggtggc 1208
          |||
Sbjct: 482 ccaagagataagatcaatcaaagacttgaanganaggaggtggc 525
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Appendix II

Poncirus trifoliata

A photo of this plant will appear in the forthcoming CD-ROM.

Common name:	Trifoliata Orange	Family:	Rutaceae
Author:	(L.) Raf.	Botanical references:	11, 109, 200
Synonyms:	Citrus trifoliata (L.), Aegle sepiaria (DC.)		
Known Hazards:	None known		
Range:	E. Asia - C. and S. China, Korea.		
Habitat:	Hedgerows[109].		
Plants For A Future Rating (1-5):	3		

Other Possible Synonyms:	From various places across the web, may not be correct. See below. P. trifoliatus[E]
Other Common Names:	From various places around the Web, may not be correct. See below. Chih [E], Chih K'O [E], Golden Apple [H], Hardy Orange [P], Hardy-orange [B], Japanese Bitter Orange [H], Karatati [E], Kou Chu [E], Trifoliata Orange [H], Wild Orange [H],
Epithets:	From a Dictionary of Botanical Epithets trifoliata = 3 leaved; trifolia = 3 leaves;
Other Range Info:	From the Ethnobotany Database China
Plant Passport required for Trade in UK/EU	From DEFRA Plant passporting. Plant Passport Required for trade in the UK/Europe.

Physical Characteristics

A deciduous shrub growing to 3m by 3m at a slow rate. It is hardy to zone 5 and is not frost tender. It is in flower from April to May, and the seeds ripen from September to November. The scented flowers are hermaphrodite (have both male and female organs) and are pollinated by Insects. The plant is self-fertile. We rate it 3 out of 5 for usefulness.

The plant prefers light (sandy), medium (loamy) and heavy (clay) soils, requires well-drained soil and can grow in nutritionally poor soil. The plant prefers acid, neutral and basic (alkaline) soils and can grow in very acid and very alkaline soils. It can grow in semi-shade (light woodland) or no shade. It requires moist soil.

Habitats and Possible Locations

Hedge, Woodland, Sunny Edge, Dappled Shade.

Edible Uses

Condiment; Fruit; Leaves.

Fruit - cooked. A bitter and acrid flavour, but it can be used to make a marmalade[3, 11, 105]. The fruit is also used to make a refreshing drink[61, 183]. The freshly picked fruit yields little juice but if stored for 2 weeks it will yield about 20% juice[183], which is rich in vitamin C. Yields of up to 14 kilos of fruit per plant have been achieved in America[160]. The fruit is 2 - 3cm wide[188], though most of this is the skin[K].

The fruit peel can be used as a flavouring[183].

Young leaves - cooked[183].

Medicinal Uses

Disclaimer

Antiemetic; Antispasmodic; Carminative; Deobstruent; Digestive; Diuretic; Expectorant; Laxative; Odontalgic; Stimulant; Stomachic; Vasoconstrictor.

The thorns are used in the treatment of toothache[218].

The stem bark is used in the treatment of colds[218].

The fruit, with the endocarp and seeds removed, is carminative, deobstruent and expectorant[176]. It is used in the treatment of dyspepsia, constipation and abdominal distension, stuffy sensation in the chest, prolapse of the uterus, rectum and stomach[176]. It is milder in effect than the immature fruit and is better used for removing stagnancy of food and vital energy in the spleen and stomach[176].

The unripe fruit is antidiarrheic, antiemetic, antispasmodic, deobstruent, digestive, diuretic, laxative, stimulant, stomachic and vasoconstrictor[147, 176, 178, 218]. It is used in the treatment of dyspepsia, constipation and abdominal distension, stuffy sensation in the chest, prolapse of the uterus, rectum and stomach, shock[176].

Other Uses

Hedge; Rootstock.

Used as a rootstock for Citrus species (oranges, lemons etc)[105, 160]. It confers an extra 3°C resistance to the cold[160].

The plant is very thorny and makes an excellent impenetrable barrier or hedge[3, 11, 29], though this barrier is not very dense[K]. The plants are very tolerant of pruning[182], they are best clipped in early summer shortly after flowering[200, 245].

Cultivation details

An easily grown plant, it succeeds in an ordinary garden soil[1], preferably well-drained[182], but prefers a fertile light sandy soil in a sunny position[11, 200]. A plant is growing and fruiting well in light woodland shade at Cambridge Botanical Gardens[K]. Plants dislike soil cultivation close to their roots and so should

either be well mulched to prevent weed growth, or hand weeded[202]. Succeeds in poor acid soils[229]. Plants also succeed in chalk-laden soils[245].

Hardy to about -15°C[184]. Plants have survived -30°C of frost without injury according to one report[11].

The bitter orange hybridizes with Citrus species and could possibly be used in breeding programmes to produce hardier forms of oranges, lemons etc[61, 160]. It could also be of value in conferring disease resistance, tolerance of poorer soils and dwarfing characteristics.

The flowers are produced on the previous years wood[200]. The whole plant, but especially the flowers[202], is strongly aromatic[182].

A very ornamental plant[1, 11], the fruits are freely formed in south-western Britain[11, 59]. A hedge at Wisley in a semi-shaded position fruits heavily in most years[K]. Another report says that warm autumns are required if the plant is to fruit freely. Fertile seed is produced after warm summers[182].

Plants are relatively short-lived, deteriorating after about 25 years[202].

Propagation

Seed - best sown as soon as it is ripe in a cold frame[200]. Cold stratify stored seed for 4 weeks and sow early spring in a greenhouse[113]. Prick out the seedlings into individual pots when they are large enough to handle and grow them on in the greenhouse for at least their first winter. Plant them out into their permanent positions in early summer.

Cuttings of half-ripe wood, June/July in a frame[1].

Scent

Flowers: Fresh

The whole plant, especially the flowers, is strongly aromatic.

Appendix 3

AA sequence of 12rhmnosyl transferase (AY048882)

```
MDTKHQDKPS ILMLPWLHAG HIAPHLELAK KLSQKNFHIY FCSTPNNLQS FGRNVEKNFS      60
SSIQLIELQL PNTFPELPSQ NQTTKNLPPH LIYTLVGAFE DAKPAFCNIL ETLKPTLVMY      120
DLFQPMAAEA AYQYDIAAIL FLPLSAVACS FLLHNIVNPS LKYPFFESDY QDRESKNINY      180
FLHLTANGTL NKDRFLKAFE LSCKFVFIKT SREIESKYLD YFPSLMGNEI IPVGPLIQEP      240
TFKVDDTKIM DWLSQKEPRS VVYASFGSEY FPSTDEIHDI AIGLLLTEVN FIWAFRLHPD      300
EKMTIEEALP QGFAEEIERN NKGMIQGWV PQAKILRHGS IGGFLSHCGW GSVVEGMVFG      360
VPIIGVPMAY EQPSNAKVVV DNGMGMVVPR DKINQRLGGE EVARVIKHVV LQEEAKQIRR      420
KANEISESMK KIGDAQMSVV VEKLLQLVKK SE                                     452
//
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P Trifoliata DNA (CF419914)

```
ggaaataatt ccagtagggc ctctaacca agaacctact ttcaaggaag atgatacaaa
gatcatggac tggctgagcc aaaaggagcc tcgttcagtc gtgtatgcat cctttggcag
tgagtacttt ccttccaagg atgaaataca tgagatagct agtggattat tgctcagcga
ggttaatttt atatgggctt tcagattaca tctgatgaa aaaatgacta tcgaggaggc
actgcctcag ggctttgctg aggagattga aaggaataat aagggaatga tagtacaagg
ttgggttccg caggctaaaa ttttaaggca tggaagcatc ggcggatttt tgagtcattg
tggttggggc tcggtagtgt aggggatggt tttcggggta ccaatcatag gtgtgccaat
ggcatatgag cagccaagca atgccaaggn ggtggttgac aatggtatgg gcatggtcgt
gccaaagagat aagatcaatc aaagacttga anganaggag gtggc
```

PRETTYSEQ of FastaSequence from 1 to 524(P. trifoliata)

BOLD = RHAMNOSYL TRANSFERASE AA SEQ from 229

```

-----|-----|-----|-----|-----|
1 gaaataattccagtagggcctctaaccaagaacctactttcaaggaagatgatacaaaag 60
1 E I I P V G P L I Q E P T F K E D D T K 20
  E I I P V G P L I Q E P T F K V D D T K

-----|-----|-----|-----|-----|
61 atcatggactggctgagccaaaaggagcctcggttcagtcgtgtatgcatcctttggcagt 120
21 I M D W L S Q K E P R S V V Y A S F G S 40
  I M D W L S Q K E P R S V V Y A S F G S

-----|-----|-----|-----|-----|
121 gagtactttccttccaaggatgaaatacatgagatagctagtggattattgctcagcgag 180
41 E Y F P S K D E I H E I A S G L L L S E 60
  E Y F P S T D E I H D I A I G L L L T E

-----|-----|-----|-----|-----|
181 gtttaattttatatgggctttcagattacatcctgatgaaaaaatgactatcgaggaggca 240
61 V N F I W A F R L H P D E K M T I E E A 80
  V N F I W A F R L H P D E K M T I E E A

-----|-----|-----|-----|-----|
241 ctgcctcaggcgtttgctgaggagattgaaaggaataataagggaatgatagtacaaggt 300
81 L P Q G F A E E I E R N N K G M I V Q G 100
  L P Q G F A E E I E R N N K G M I V Q G

-----|-----|-----|-----|-----|
301 tgggttccgcaggctaaaattttaaggcatggaagcatcggcggatttttgagtcattgt 360
101 W V P Q A K I L R H G S I G G F L S H C 120
  W V P Q A K I L R H G S I G G F L S H C

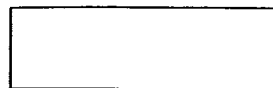
-----|-----|-----|-----|-----|
361 ggttggggctcggtagttaggggatggttttcggggtaccaatcataggtgtgccaatg 420
121 G W G S V V E G M V F G V P I I G V P M 140
  G W G S V V E G M V F G V P I I G V P M

```

```
-----|-----|-----|-----|-----|
421 gcatatgagcagccaagcaatgccaaaggnggtgggttgacaatggtatgggcatggtcgtg 480
141 A Y E Q P S N A K E V V D N G M G M V V 160
    A Y E Q P S N A K V V V D N G M G M V V
```

```
-----|-----|-----|-----|
481 ccaagagataagatcaatcaaagacttgaanganaggaggtggc 524
161 P R D K I N Q R L E R K E V 174
    P R D K I N Q R L G G E E V
```

Appendix 4



NCBI Conserved Domain Search

[New Search](#)

[CDD](#)

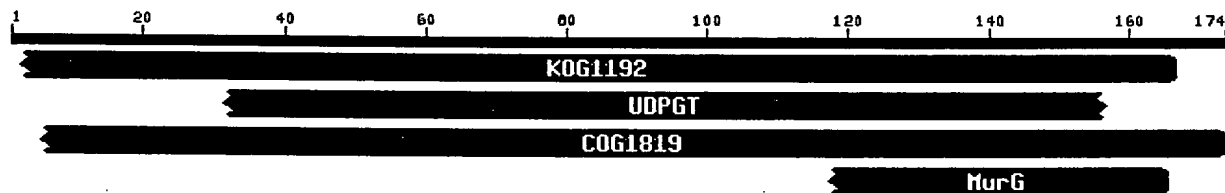
[Help?](#)

RPS-BLAST 2.2.6 [Apr-09-2003]

Query= local sequence:
(174 letters)

Database: cdd.v1.63
16,482 PSSMs; 5,198,197 total columns

Click on boxes for multiple alignments



Domain Relatives

☐ .. This CD alignment includes 3D structure. To display structure, download [Cn3D!](#)

PSSMs producing significant alignments:

Score

(bits)

E

value

[gnl|CDD|18981](#)

KOG1192, KOG1192, UDP-glucuronosyl and UDP-glucosyl transferas...

[105](#)

5e-24

gnl|CDD|22944

pfam00201, UDPGT, UDP-glucuronosyl and UDP-glucosyl transferase

70.4

2e-13

gnl|CDD|11529

COG1819, COG1819, Glycosyl transferases, related to UDP-glucur...

64.3

1e-11

gnl|CDD|10576

COG0707, MurG, UDP-N-acetylglucosamine:LPS N-acetylglucosamine...

35.3

0.007

gnl|CDD|18981, KOG1192, KOG1192, KOG1192, UDP-glucuronosyl and UDP-glucosyl transferase
[Carbohydrate transport and metabolism, Energy production and conversion]

CD-Length = 496 residues, only 32.9% aligned

Score = 105 bits (263), Expect = 5e-24

Query: 2 IIPVGPLIQEPTFKVDDTKIMDWLS--QKEPRSVVYASFGSE---YFPSTDEIHDAIGL
56

Sbjct: 247 VIPIGP-LHVKDSKQKSPLPLEWLDILDESRSVYISFGSMVNSADLP EEQKKELAKAL
305

Query: 57 -LLTEVNFIWAFRLHPDEKMTIEEALPQGFAEEIERNNKGMIVQ-GWVPQ-AKILRHGSI
113

Sbjct: 306 ESLQGVTFWLKYRPDDSIY-----FPEGLPNRGRGNVVL SKWAPQNDLLLDHPAV
355

Query: 114 GGFLSHCGWGSVVEGMVFGVP IIGVPMAYEQPSNAKVVDNGMGMVVPRDKINQ 167

Sbjct: 356 GGFVTHGGWNSTLESIYSGVPMVCVPLFGDQPLNARLLVRHGGGGVLDKRD LVS 409

gnl|CDD|22944, pfam00201, UDPGT, UDP-glucuronosyl and UDP-glucosyl transferase.

CD-Length = 501 residues, only 22.2% aligned

Score = 70.4 bits (172), Expect = 2e-13

Query: 31 RSVVYASFGSEYFPSTDE-IHDAIGLLLTEVNFIWAFRLHPDEKMTIEEALPQGFAEEI
89

Sbjct: 276 HGVVVFSLGSMVSNIPEEKANEIASALAQIPQKVLWRFD-----G TKPST---L
321

Query: 90 ERNNKGMIVQGWVPQAKILRHGSIGGFLSHCGWGSVVEGMVFGVP IIGVPMAYEQPSNAK
149

Sbjct: 322 GNNT---RLVKWLPQNDLLGH PKTRAFVTHAGSNGVYE AICHGVPMVGMPLFGDQMDNAK
378

Query: 150 VVVDNGMG 157

Sbjct: 379 HMEAKGAA 386

gnl|CDD|11529, COG1819, COG1819, Glycosyl transferases, related to UDP-glucuronosyltransferase
[Carbohydrate transport and metabolism / Signal transduction mechanisms]

CD-Length = 406 residues, only 36.7% aligned

Score = 64.3 bits (156), Expect = 1e-11

Query: 5 VGPLIQEPTFKVDDTKIMDWLSQKEPRSVVYASFGSEYFPSTDEIHDAIGLLLTE-VNF
63
Sbjct: 214 IGPYIGPLLGEAAN-ELPYWIPADRP--IVYVSLGTV--GNAVELLAIVLEALADLDVRV
268

Query: 64 IWAFLRLHPDEKMTIEEALPQGFAEEIERNNKGMIVQGWVPQAKILRHGSIGGFLSHCGWG
123
Sbjct: 269 I----VSLGGARDTLVNVDPNV-----IVADYVPQLELLPRA--DAVIHHGGAG
311

Query: 124 SVVEGMVFGVPIIGVPMAYEQPSNAKVVVDNGMGMVVPDKINQRLGGEEV 174
Sbjct: 312 TTSEALYAGVPLVVIPDGADQPLNAERVEELGAGIALPFEELTEERLRAAV 362

gnl|CDD|10576, COG0707, MurG, UDP-N-acetylglucosamine:LPS N-acetylglucosamine transferase [Cell envelope biogenesis, outer membrane]

CD-Length = 357 residues, only 15.1% aligned

Score = 35.3 bits (81), Expect = 0.007

Query: 117 LSHCGWGSVVEGMVFGVPIIGVPMAY----EQPSNAKVVVDNGMGMVVPDKIN 166
Sbjct: 257 ISRAGALTIAELLALGVPAILVPYPPGADGHQEYNAKFLEKAGAALVIRQSELT 310